REMARKS/ARGUMENTS

The Applicants respectfully submit this amendment in connection with a *Request for Continued Examination (RCE)*.

Upon entry of this amendment, claims 82-96 will be pending in the application and presented for examination. Claims 1-81 have been canceled without prejudice to subsequent revival. Applicants reserve the right to prosecute these canceled claims in a continuation application. Claim 82 has been amended. Dependent claims 83-96 have been newly added. No new matter was added by this amendment. Entry of the amendment, reconsideration of the rejection, and allowance of claims 82-96 are respectfully requested.

The Amendment

In order to expedite prosecution of the application and advance the case toward allowance, the claims have been amended.

More specifically, claim 82 has been amended to specify that the tissue remodeling and/or angiogenic substance is administered to the "gingiva". Support for this amendment can be found in the specification, for example, on page 3, paragraph [0009], lines 25-32.

New claim 83 finds support, for example, on page 2, paragraph [0006], lines 17-19.

New claim 84 finds support, for example, on page 3, paragraph [0009], lines 25-31.

New claim 85 finds support, for example, on page 4, paragraph [0011], lines 17-20.

New claim 86 finds support, for example, on page 4, paragraph [0011], lines 18 and 20.

New claim 87 finds support, for example, on page 8, paragraph [0031], lines 1-3.

New claim 88 finds support, for example, on page 4, paragraph [0010], line 2.

New claim 89 finds support, for example, on page 5, paragraph [0013], line 11 and page 6, paragraph [0016], line 6.

New claims 90 and 91 find support, for example, on page 4, paragraph [0011], lines 24-29 and page 6, paragraph [0016], lines 20-22.

New claims 92, 93 and 94 find support, for example, on page 3, paragraph [0009], lines 18, 19, 21 and lines 25-32.

New claim 95 finds support, for example, on page 2, paragraph [0006], lines 21-23.

New claim 96 finds support, for example, on page 1, paragraph [0003], lines 13-14 and paragraph [0004], lines 23-27.

Information Disclosure Statement

The Office Action indicates that the information disclosure statement (IDS) filed on May 7, 2004 has not been considered because it lacks a statement as specified in 37 C.F.R. §1.97(e) or the fee set forth in 37 C.F.R. §1.17(p).

The supplemental IDS was filed on May 5, 2004, which date was less than 3 month after the mailing of the *International Search Report* on April 19, 2004. The omission of the statement under 37 C.F.R. §1.97(e) was unintentional. Enclosed herewith is the supplemental IDS and a copy of the International Search Report for the convenience of the Examiner. Applicants respectfully request that the Examiner consider the IDS.

Rejection Under 35 U.S.C. §101

Claim 78 is rejected under 35 U.S.C. §101 as being allegedly drawn to non-statutory subject matter. Claim 78 has been canceled. Thus, the rejection is moot.

Rejection Under 35 U.S.C. §102

Claim 78 remains rejected under 35 U.S.C. §102(b), as being allegedly anticipated by Nicozisis *et al.* Claim 78 has been canceled. Thus, the rejection is moot.

Rejection Under 35 U.S.C. §103

Claims 1-8, 10-19, 22-29, 31-40, 56-64, 66-75, 79-82 remain rejected under 35 U.S.C. §103(a), as allegedly being obvious over Nicozisis *et al.*

The Office Action indicates that based on the suggestions provided by Nicozisis *et al.*, it would have been obvious to one skilled in the art at the time the invention was made to administer relaxin during orthodontic treatment. The Examiner refers to several passages in Nicozisis *et al.*, wherein relaxin and its effects on the periodontium are addressed.

To the extent that the rejection applies to the claims as amended, the rejection is respectfully traversed.

Claim 82 has been amended to specify that the claims refer to a method of preventing relapse in a patient, wherein force is applied to at least one tooth, and a tissue remodeling and/or an angiogenic substance is administered to the patient to promote remodeling of the "gingiva," wherein the substance is administered to the patient at "a dose from about 1 ng to about 500 μg per day".

Nicozisis *et al.* do not teach or suggest a method of preventing relapse wherein a substance such as relaxin is applied to the gingiva at a dose of about 1 ng to about 500 μg per day in combination with applying force. To the contrary, Nicozisis *et al.'s* studies focus primarily on the effects of relaxin onto the craniofacial sutures and the periodontium or periodontal ligament (PDL). In fact, Nicozisis *et al.* indicate upfront that their report is "a report of an exploratory study of how the hormone relaxin might modulate the remodeling of connective tissue within the craniofacial sutures and periodontal tissues" (see first sentence of Abstract).

It is well established that all the limitations of the claims must be disclosed by the combination of references (or reference) cited in the prior art in order to establish *prima facie* obviousness (MPEP §2143.03). The amended claims specify that in the instant method, a substance such as relaxin is administered to the patient's gingiva at a dose of about 1 ng to about 500 μg per day in combination with applying force. Nicozisis *et al.* do not teach or suggest such a method and certainly do not teach all the limitations of the amended claims. The Office Action indicates on page 7 that one cannot show nonobviousness by attacking references individually

where the rejection is based on a combination of references. However, if Nicozisis *et al.* is combined with Kuo *et al.* and/or Korostoff *et al.* and/or Burgio, the references still do not teach all of the limitations of the amended claims.

On page 6 of the Office Action, the Examiner asserts that "...throughout the Discussion beginning on page 196, Nicozisis *et al.* recite that the effects of relaxin are not limited to the craniofacial structure, but also to the periodontium and thus would have applications in the field of orthodontics." The Examiner is correct in pointing out that Nicozisis *et al.* focused their study on the craniofacial structure and the periodontium.

For example, in their experimental section, Nicozisis *et al.* first determine the presence and effects of relaxin in the cranial sutures (see page 194, second column, last paragraph). Secondly, Nicozisis *et al.* determine the effects of relaxin on the arrangement of collagen fibers in the PDL (see page 195, first column, last paragraph). Nicozisis *et al.* could not prove the presence of relaxin in the PDL because they could not section the tissues. Instead, they found that the PDL of the samples treated with relaxin demonstrated an irregular organization and loose arrangement from tooth to bone surface, and that the fibers lacked directionality and appeared short and curled (see Figure 2 on page 195). However, there is no data suggestion or discussion of gingiva.

In comparison, the instant application includes specific embodiments focused on the gingiva, *e.g.*, the specification states the following on page 8, paragraph [0031]:

> In some cases, it will be desired to deliver the drug into the gingival margin which is the line or groove along the gingiva-tooth interface. Substances may be applied as part of formulations which are delivered over the gingiva and/or into the sulcus. In some instances, it may be desirable to plant small substance delivery structures directly into the sulcus in a manner analogous to the delivery of antibiotics using systems, such as the PerioChip® available from Dexcel Pharma.

The gingiva is also referred to as the gums, *i.e.*, the soft tissue surrounding the tooth (see Figure 1 in Appendix A attached hereto). It is generally known in the art that the gingival tissues are located supracrestal, *i.e.*, not near the root of a tooth. Overall, periodontal diseases are a group of conditions that affect the gums (*i.e.*, gingiva) and/or destroy the

underlying supporting tissues (*i.e.*, alveolar bone, cementum, and periodontal ligament). Periodontal diseases are broadly classified into gingivitis and periodontitis. For example, gingivitis is an inflammation of the gums that can bleed upon touching. Because inflammation of the gums must occur before the bone is destroyed (as in periodontal disease), gingivitis is often the precursor to periodontal disease. Gingivitis may persist for years before the inflammatory process allows destruction of the bone to occur, thus, leading to periodontal disease. Some orthodontic techniques are focused mainly on the gingiva. For example, *Gingivectomy* is the surgical removal of gingiva while *Gingivoplasty* is a surgical procedure to reshape or repair the gingiva. Both techniques may be employed to treat patients who suffer from gingivitis. Along those lines, *Supracrestal Fiberotomy* is known to be a surgical technique to divide the free gingival and transseptal fibers around rotated teeth. More specifically, *Circumferential Supracrestal Fiberotomy* (CSF) is a technique used to minimize rotational relapse.

In contrast, the periodontal ligament (PDL) refers to the fibrous tissue that occupies the space between tooth root and alveolar bone (see Figure 2 in Appendix B attached hereto), providing a ligamentous physical attachment of the tooth to the bone and nourishment to the cementum surface of the root and the cells that permit tooth movement. Thus, the PDL essentially holds the tooth in the bone and is composed of connective tissue. On a radiograph, the healthy PDL appears as a fine, black, radiolucent line next to the root surface. On its outer side, is the lamina dura, the bone lining the tooth socket. With disease, the PDL space may appear at varying thicknesses, which can show that the disease involvement is not consistent around the entire root. Periodontitis occurs when the inflammatory infiltrate spreads from the gingiva into the underlying supporting tissues, resulting in periodontal pockets and alveolar bone loss. A periodontal pocket is an abnormal deepening of the gum crevice that is caused when disease and infection destroy the PDL that attaches the gum to the tooth and the underlying bone. Tooth looseness can occur later in the disease process.

As the Examiner will appreciate, there are distinct differences between the gingiva and the PDL. Such differences are further illustrated by how oral fibroblasts respond to force. For example, oral fibroblasts in the gingiva respond differently to applied force then oral

fibroblasts in the PDL. The effects of centrifugal force can mimic orthodontal force as discussed in Redlich *et al.* (see **(1)** Redlich, M., *et al., J. Periodontal Res.,* 2004. 39(1): p. 27-32; and **(2)** Redlich, M., *et al., Arch. Oral. Biol.,* 1998. 43(4): p. 313-316). In (1) and (2) it is shown that the effects of centrifugal force differed in PDL fibroblasts and gingival fibroblasts. PDL fibroblasts responded with increased levels of collagenase (*i.e.,* a matrix metalloproteinase-1 (MMP-1)) while showing no change in the levels of collagen or tissue inhibitors-1 and -2 (TIMPs) as indicated in the abstract of (1). In contrast, gingival fibroblast responded to applied force with decreased levels of collagenase and increased levels of collagen-1 as indicated in the abstract of (2). Thus, the differences are apparent, the PDL showed increased collagenase which would essentially support remodeling of the tissue while the gingiva decreased collagenase which suggests increased stability of the tissue. In light of what is known in the art, the skilled artisan would certainly understand that any findings about the PDL would not necessarily correlate to similar findings in the gingiva. To the contrary, as shown above, the opposite could be true.

Notably, in their discussion section (see page 196, second column, last paragraph), Nicozisis *et al.* state that "The purpose of this study was to explore the possibilities of how the hormone known as relaxin might modulate the remodeling of the connective tissue within the craniofacial sutures and the periodontium." They refer thereby to the PDL. In fact, they state on page 197 (second column, second paragraph) that "the histological evidence in this study exhibits positive effects of relaxin on the connective tissue within the PDL and cranial sutures". They further state that their "findings could be strengthened by the use of specific inhibitors to identify which specific enzymes are responsible for the degradation of the connective tissue within the craniofacial complex and periodontium." (See page 198, first column, third paragraph). Again, they refer thereby to the sutures and PDL.

Respectfully, the skilled artisan could not arrive at the instant method as recited by the amended claims by looking to Nicozisis *et al.* or by looking to Nicozisis *et al.* in combination with Kuo *et al.* and/or Korostoff *et al.* and/or Burgio. Neither Nicozisis *et al.* alone nor any of the other references make any suggestion of a method that could prevent relapse by administering force and applying a specific dose of a substance like relaxin to the gingiva. Particularly, since Nicozisis *et al.* focused their study on the PDL and the Examiner has provided

no evidence that these findings could be equally applicable to the gingiva, one of skill would not be motivated to prevent relapse by administering force and applying a substance like relaxin to the gingiva. MPEP §2141 states that the references must be viewed without the benefit of *impermissible hindsight* vision afforded by the claimed invention... (see Basic Considerations which apply to Obviousness Rejections). The Examiner must forget what the instant invention has taught in order to evaluate if the cited references could suggest what is currently claimed. Since there is no teaching or even suggestion in any of the references of a method of preventing relapse by treating the gingiva with a substance like relaxin at a dose from about 1 ng to about 500 μg per day in combination with applying force, there would simply be no motivation to combine the references.

In light of the above amendments and remarks, Applicants respectfully request that the rejection of claim 82 under 35 U.S.C. §103(a), be withdrawn and the application be sent to issue.

Claims 9, 30 and 65 are rejected under 35 U.S.C. §103(a), as being allegedly obvious over Nicozisis *et al.* as applied to claims 8, 29 and 64, and further in view of Kuo *et al.* (U.S. Patent No. 6,607,382).

Claims 9, 30 and 65 have been canceled. Thus, the rejection is moot.

Claims 20, 21, 41, 42, 76 and 77 are rejected under 35 U.S.C. §103(a), as being allegedly obvious over Nicozisis *et al.* as applied to claims 1, 22 and 56 and further in view of Korostoff *et al.* (U.S. Patent No. 4,153,060).

Claims 20, 21, 41, 42, 76 and 77 have been canceled. Thus, the rejection is moot.

Claims 43-46, 48 and 51-55 are rejected under 35 U.S.C. §103(a), as being allegedly obvious over Kuo *et al.* in view of Nicozisis *et al.*

Claims 43-46, 48 and 51-55 have been canceled. Thus, the rejection is moot.

Claims 43-47, 49 and 50 are rejected under 35 U.S.C. §103(a), as being allegedly obvious over Burgio (U.S. Patent No. 6,322,360) in view of Nicozisis *et al.*

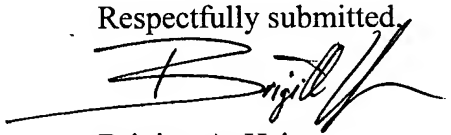
Claims 43-47, 49 and 50 have been canceled. Thus, the rejection is moot.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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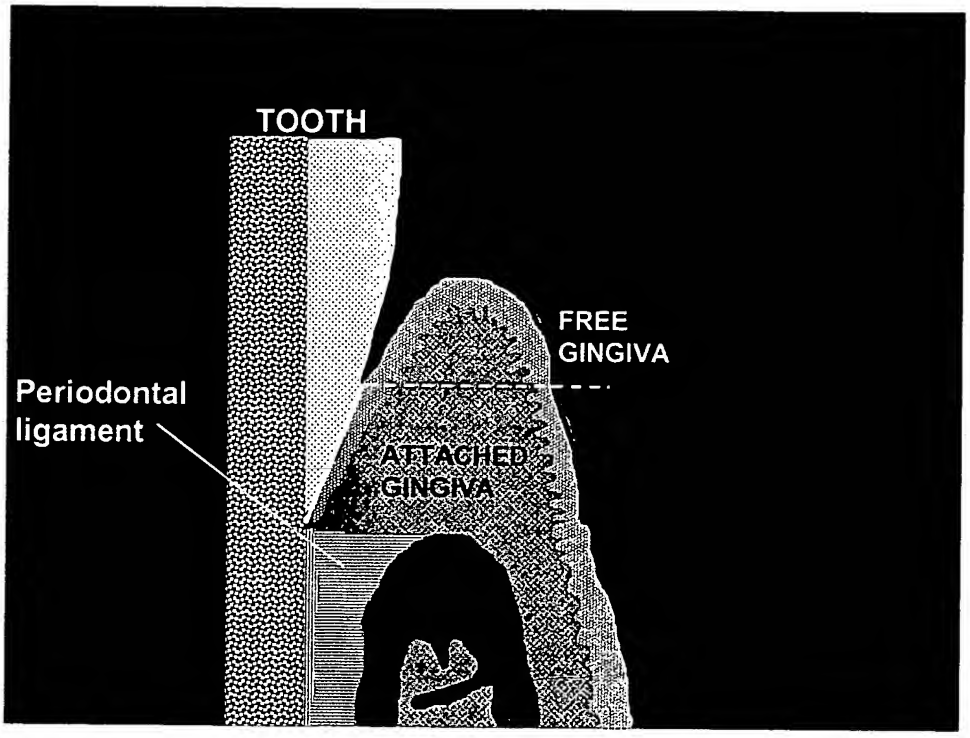
TOOTH
FREE
GINGIVA
Periodontal
ligament
ATTACHED
GINGIVA

FIGURE 2

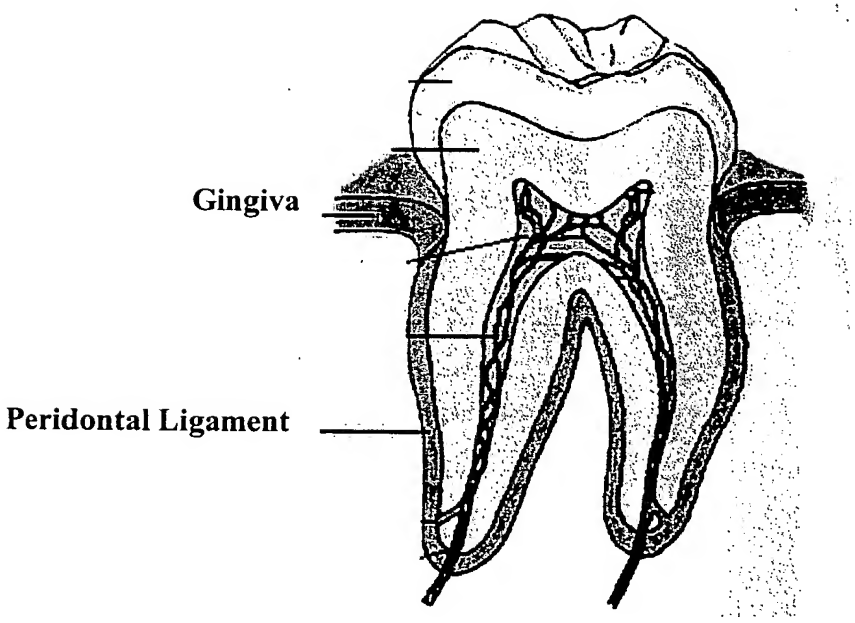

FIGURE 1

J Periodont Res 2004; 39; 27–32
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JOURNAL OF PERIODONTAL RESEARCH

The effect of centrifugal force on mRNA levels of collagenase, collagen type-I, tissue inhibitors of metalloproteinases and β-actin in cultured human periodontal ligament fibroblasts

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Redlich M, Roos H, Reichenberg E, Zaks B, Grosskop A, Bar Kana I, Pitaru S, Palmon A. The effect of centrifugal force on mRNA levels of collagenase, collagen type-I, tissue inhibitors of metalloproteinases and β-actin in cultured human periodontal ligament fibroblasts. J Periodont Res 2004; 39; 27–32. © Blackwell Munksgaard, 2004

Background: The aim of orthodontic treatment is to relocate teeth abnormally positioned in the jaws. This is achieved by application of continuous force on the tooth, which is immediately being sensed by the periodontal ligament (PDL), bone and the gingiva. Since the bony response is mediated by the PDL, tooth movement is primarily a PDL phenomenon.

Objectives: Thus, the purpose of the present study was to evaluate the direct effect of force (excluding the *in vivo* tissue response) on the molecular level of matrix metalloproteinase-1 (MMP-1) and collagen type-I (Col-I) in human PDL fibroblasts.

Methods: PDL cell culture flasks were centrifuged for 10, 20, 30, 60, 90 and 120 min by horizontal microplate rotor. The effect of force on mRNA levels of β-actin, MMP-1, Col-I, tissue inhibitors-1 and -2 (TIMPs) genes was analyzed by RT-PCR.

Results: The results showed that force had no effect on the mRNA levels of β-actin during the first 90 min of application of force, indicating for the first time the use of β-actin gene as an internal invariant control. It increased the mRNA levels of MMP-1 while almost no effect on Col-I and TIMPs was observed.

Conclusions: The results indicate that PDL remodeling following application of orthodontic force could be partly attributed to the direct effect of the force on MMP-1 gene expression in fibroblasts.

*Senior authors, contributed equally to this study.

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Key words: gene expression; matrix metalloproteinase-1; orthodontic force; periodontal ligament

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The periodontal ligament (PDL) provides anchorage and support to the functioning tooth. Its extracellular matrix (ECM) consists predominantly of collagen type-I (Col-I), whereas fibroblasts are the most abundant cells in the PDL (1). The turnover of the collagen fibers in the PDL is very rapid (2). This steady-state homeostasis implicates towards intensive and subtle transcriptional and translational regulation of the collagens and the matrix metalloproteinases (MMPs) genes. MMPs, a family of zinc-dependent enzymes, have the capacity to degrade almost all components of the ECM (3). Within this family of enzymes, MMP-1 initiates cleavage of fibrillar collagens at a single site, a prerequisite process for further degradation of the cleaved fragments by other MMPs.

The aim of orthodontic treatment is to relocate teeth abnormally positioned in the jaws. This is achieved by application of continuous mechanical force on the tooth, which immediately affects the PDL, the bone and the gingiva. The immediate effect of an *in vivo* orthodontic force is dual: (i) injury to the PDL and a subsequent wound-healing process and (ii) exertion of mechanical force on the cell. Thus, the cells in the PDL simultaneously respond to two different events: ECM damage and mechanical force. The latter is assumed to be sensed by cell membrane components and then transduced intracellulary into biological signals.

The dental attachment apparatus, which responds to orthodontic forces, is exposed to pressure-type forces (in the direction of tooth movement) and tension-type forces (the opposite side of the pressure) leading to remodeling of the PDL, the bone and the gingiva which enables the transposition of the tooth (4).

Recently, we showed that following application of force the *in vivo* molecular equilibrium between collagen synthesis and degradation in the gingiva is disturbed (5). Both pressure and tension vectors of the force cause a significant increase in the mRNA levels of MMP-1, as well as in its interstitial activity.

Separating the dual effect of the orthodontic force (tissue injury and cell–force interaction) is impossible in an *in vivo* system and therefore only an *in vitro* model is capable of simulating genuine cells–force interactions. Also, the *in vitro* model should differentiate between pressure and tension types of force. In an earlier study we established an *in vitro* pressure model by centrifugation of cell culture (6). The advantage of the centrifugal pressure model, as well as the other *in vitro* tension systems (7, 8), is that it evaluates the direct effect of pressure or tension on the cells excluding the *in vivo* tissue response to the force. Consequently, direct correlation is established between pressure/tension force and phenotypic cellular response. Since the bony response is mediated by the PDL, tooth movement is primarily a PDL phenomenon. Thus, the purpose of the present study was to evaluate the molecular MMP-1 and Col-I interactions of human PDL fibroblasts subjected to an *in vitro* continuous pressure type of mechanical force.

Materials and methods

Cell culture

Human PDL fibroblasts were isolated from healthy PDL of premolar teeth of individuals undergoing tooth extraction for orthodontic treatment in accordance with the method of Somerman *et al.* (9), with minor modifications as we previously published (10, 11). All the patients gave informed consent before providing the samples. Healthy periodontal tissue was removed from the center of the root surface with a surgical scalpel. The tissue was minced, and then transferred to plastic Leighton tubes (Costor, Cambridge, MA, USA). The explants were cultured in α-MEM supplemented with 10% fetal calf serum, 50 U/ml penicillin G and 50 μg/ml streptomycin (henceforth denoted standard medium) with changing medium every 2 or 3 days. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When the cells growing out from the explants had reached confluence, they were separated by treatment with 0.05% trypsin–0.53 mM ethylenediaminetetraacetic acid (EDTA), collected by centrifugation, and cultured on culture plastic dishes containing the standard medium until confluency. The cells were then trypsinized at 1 : 3 split ratio. Experiments were carried out with cells from the second (p2) or third (p3) passages. In this study, for each experiment, we used explants from at least three different volunteers.

Application of centrifugal force *In vitro*

Cell culture flasks ($n > 3$) were centrifuged at 167 *g* for 10, 20, 30, 60, 90 and 120 min by horizontal microplate rotor as previously described (6). This model is based on application of a constant pressure centrifugal force at a magnitude of 33.5 g/cm^2, which resembles clinical orthodontic force (4).

The calculation of force is based on the equation:

$$P=(m\times r\times \text{r.p.m.}^2\times\pi^2)/(A\times 9.8\times 900),$$

where P = kg pressure per cm^2 of cells, m = mass of medium (0.005 kg), r = radius (0.15 m), r.p.m. = revolu-evolution/min (1000), A = area of contact between medium and cells (25 cm^2).

Trypan blue cell vitality exclusion test

Trypan blue cell vitality exclusion test was performed as we previously described (6).

Cell collection and RNA extraction

Monolayer cell cultures were suspended in TRI-REAGENT (Molecular Research Center, Cincinnati, USA). Total RNA was extracted as previously described (10, 11).

Determination of mRNA levels of Col-1, MMP-1, TIMP-1 and TIMP-2 by RT-PCR

To measure mRNA levels of β-actin, Col-I, MMP-1, tissue inhibitors-1 and -2 (TIMP-1 and TIMP-2), semi-quantitative RT-PCR analysis was performed for each gene transcript as previously described (5, 10, 11). Primer sequences and the optimal conditions for each set of primers derived

from calibration curves are presented in Table 1 and Figs 1(a and b). Actin was used as an internal invariant control subsequent to providing data about its non-dependent expression following application of force (see Results). When calculating the effect of force we normalized the results to actin mRNA levels and therefore the effect of force is relative to housekeeping gene actin.

Statistical analysis

For statistical analysis, student's unpaired *t*-test was used for individual matched-group comparisons. Data are expressed as means ± SE. Values of $p < 0.05$ were considered to be statistical significant.

Results

The effect of centrifugal force on mRNA levels of β-actin

Because no data is available on the effect of pressure on mRNA levels of β-actin, in initial experiments, the mRNA levels of β-actin expressed by PDL cells in culture were determined following application of different durations of centrifugal force and compared to control (non-centrifuged) cultures. In the first 90 min of application of centrifugal force almost no change in the β-actin mRNA levels was found (Fig. 2), thereafter at 120 min a decrease of 20% below control levels was detected (Fig. 2). By that time (120 min), 80% cell vitality was shown by the vitality exclusion test. The 18S ribosomal RNA stained by ethidium bromide on agarose gel paralleled cell vitality results. The average ($n = 57$) experimental to control β-actin mRNA levels in the first 90 min was calculated to be 0.99 ± 0.13.

The effect of centrifugal force on mRNA levels of MMP-1, Col-I, TIMP-1 and TIMP-2

To monitor the kinetic effect of force on PDL cells, cell culture flasks were subjected to centrifugal force for 10, 20, 30, 60 and 90 min and mRNA levels were determined. After 30 min the mRNA levels of MMP-1 (compared to control and normalized to β-actin) increased significantly ($p < 0.05$) to a peak level of 2.6-fold. Thereafter, at 60 min, the mRNA levels decreased but still remained higher than the control levels and at 90 min mRNA levels decreased almost to control levels (Fig. 3).

The mRNA levels of Col-I, TIMP-1 and TIMP-2, showed the same pattern as MMP-1, but in a lesser degree: in the first 60 min of application of force there was an increase to peak levels, thereafter at 90 min there was a decrease to almost control levels. The peak levels for Col-I and TIMP-1 were found to be about 1.25, and 1.6 for TIMP-2.

Discussion

Actins constitute a family of highly conserved proteins found in all eukaryotic cells. Whitin this family, α-actins are the muscle actins involved in muscle contraction and β-actins are known as the cytoplasmic type found in many non-muscle cells (12). The β-actin filaments are involved in cell motility, cell morphology and various cytoskeletal functions such as transduction of extracellular stimuli into cellular responses (13). Although β-actins are commonly used as invariant internal control in many mRNA expression studies, we could not find a conclusive report showing that mechanical stress does not affect mRNA levels of fibroblasts cytoplasmatic β-actin.

The results of the present study show no changes in mRNA levels of β-actin during the first 90 min of application of pressure on PDL fibroblasts. This finding was also accompanied with similar amounts of intact 18S ribosomal RNA and cell vitality levels, emphasizing that β-actin mRNA levels followed cell vitality and were not altered by the force. It verifies, for the first time, the use of β-actin gene as invariant control in fibroblasts reponse to mechanical loading.

A recent study (14), in which tensile mechanical loading was applied on tendon cells, showed no changes in the amount of cytoskeletal actin stress fibers at the protein level. However, a significant spatial organization of these fibers was inflicted by the force.

ECM remodeling is a highly regulated complex process, which control the homeostasis and repair of connective tissues such as the PDL following application of mechanical orthodontic force. The initial effect of the

Table 1. Sequences of primer sets

Primers	Sequences	Annealing (°C)	Mg (mM)	Length (bp)	Reference
MMP-1	5′-TGGGAGCAAACACATCTGA-3′ (sense) 5′-ATCACTTCTCCCCGAATCGT-3′ (anti-sense)	48	2.5	560	Aust *et al.* (24)
Collagen type-1α1	5′-CTGGCAAAGAAGGCGGCAAA-3′ (sense) 5′-CTCACCACGATCACCACTCT-3′ (anti-sense)	62	1.0	502	Spotila *et al.* (25)
TIMP-1	5′-ATCCTGTTGTTGCTGTGGCTGATAG-3′ (sense) 5′-TGCTGGGTGGTAACTCTTTATTTCA-3′ (anti-sense)	60	1.0	689	Alvares *et al.* (18)
TIMP-2	5′-GGAAGTGGACTCTGGAAACGACATT-3′ (sense) 5′-CTCGATGTCGAGAAACTCCTGCTTG-3′ (anti-sense)	62	2.0	495	Nomura *et al.* (26)
Actin	5′-GAGACCTTCAACACCCCAGCC-3′ (sense) 5′-GGCCATCTCTTGCTCGAAGTC-3′ (anti-sense)	62	1.0	311	Palmon *et al.* (27)

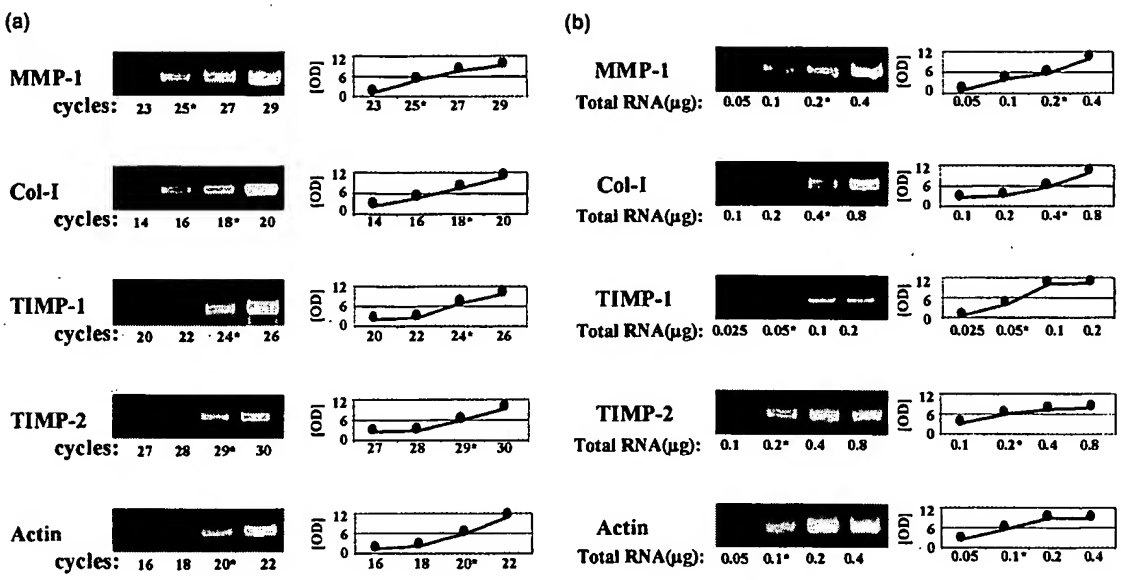

Fig. 1. Calibration curves for MMP-1, Col-I, TIMP-1, TIMP-2, and β-actin. Total RNA (1 μg) was reverse transcribed using oligo $(dt)_{15}$ primer. cDNA was amplified by PCR using the relevant primers. (b) input total RNA amplified with the PCR cycles determined by (a). Optimal conditions used for each amplification primer are marked with *.

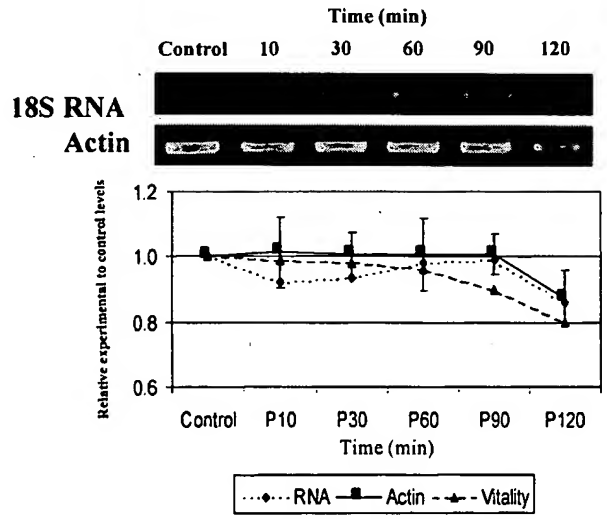

Fig. 2. 18 S RNA, actin PCR-amplified mRNA levels and cell vitality of control and force-affected cells. Upper panel show ethidium bromide stained agarose gel of 18S RNA and actin PCR-amplified bands. Lower graphs show relative optical density (OD) to control of 18S RNA and actin PCR-amplified bands at each time point and cell vitality data.

orthodontic pressure on PDL is tissue damage, which will later affect the adjacent alveolar bone (4). Temporal concerted cells–ECM interactions, as well as the response of cells to extracellular signals are essential in tissue remodeling. Consequently, regulated response of ECM proteins (collagens) on the one hand and MMPs and their inhibitors, TIMPs, on the other hand is required for this process to occur properly (15). The present study focused on the molecular response of human PDL fibroblasts to centrifugal pressure, which simulates the commonly used orthodontic force. The conspicuous finding of this study is the time-dependent up-regulation of mRNA levels of MMP1 in the first 30–60 min of application of force. This effect was time limited and was followed by a decrease of MMP-1 mRNA almost to control levels after 90 min of force application. This time-limited phenomenon, which was also found *in vivo* (5), highlights the importance of not only MMP-1 up regulation in response to force but also the control-limiting mechanism of MMP-1 gene necessary for maintaining proper tissue integrity. Interestingly, the time course of MMP-1 induction and normalization in the *in vitro* model was by far more rapid than in the *in vivo* model, which corresponds to the high turnover rate of PDL cell culture model used in this study (2). In the present study we focused on the immediate response, bearing in mind that it is a part of a broader phenomenon. We are currently investigating longer force induction periods.

To prove the principle that centrifugal pressure in an *in vitro* model induces changes in the pathway/s of extracellular trigger to mRNA transcription we used the semi-quantitative RT-PCR assay that we previously established (6). The results found are within the scope of this method. Other methods, such as real-time PCR or zymography, may be needed in the future to further quantitatively characterize this phenomenon. However, such an accurate determination is not needed for utilizing this model to elu-

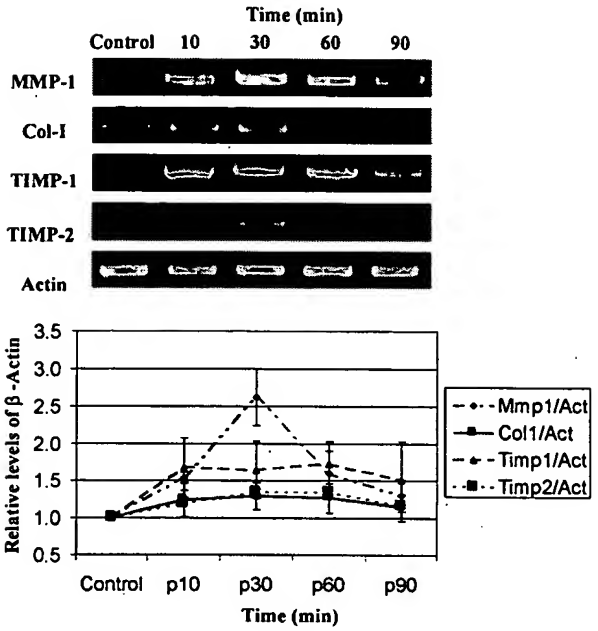

Fig. 3. Semi-quantitative RT-PCR assay of MMP-1, Col-I, TIMP-1 and TIMP-2 following application of force. Upper panels show representative set of PCR bands after gel electrophoresis. Lower graphs show the ratio between OD of force to OD of control at each time point after normalization to β-actin (for details see text). Each time point represents the mean ± SE.

cidate the cellular underlying mechanisms induced by centrifugal pressure.

The increase in MMP-1 mRNA, concomitant with no changes in mRNA levels of TIMPs indicate that elevated levels of MMP-1 protein might be synthesized leading to increased PDL remodeling. A similar process occurred in the *in vivo* model (5), in which orthodontic pressure caused significant induction of gingival mRNA levels of MMP-1 followed by increased protein activity.

Since an inevitable consequence of orthodontic intervention is that of tissue injury and subsequent inflammatory response, one may assume that PDL degradation is the result of the inflammatory response, during which collagenase is highly expressed by polymorphonuclear cells, macrophages and by fibroblasts that invade the injured area (16). The major finding of the present study that force triggers MMP-1 gene expression indicates that PDL remodeling, following application of force, is the cumulative result of both the force affecting the cells and of the inflammatory process.

The responsiveness of MMP-1 gene to force, as shown in the present work, supports previously described changes in the expression of MMP-1 genes under the influences of various cellular and extracellular factors such as alteration in actin cytoskeleton (17), growth factors (18), chemical agents (19) and physical stress (20). It also corresponds to a recent *in vitro* tension model in which both PDL and gingival human fibroblasts over expressed mRNA encoding for MMP-1 (21).

The precise regulation of MMP gene expression in relation to collagen gene expression is critical for tissue repair and homeostasis. Non-coordinated changes in the expression of each of the two genes may lead to pathologic events such as keloids formation due to over expression of Col-I (22) or to certain ulcerative skin lesions due to over expression of MMP-1 (23). Therefore, at the molecular level, the amount and timing of expression of each of these genes is essential for proper tissue repair. However, the relatively short duration of centrifugation in this study (due to cell death), as compared to the *in vivo* model (5), does not provide sufficient data for evaluating the force interdependent changes between MMP-1 and Col-I mRNAs.

This issue, as well as tracking possible pathways involved in the mechano-signal transduction processes, is the objective of our current investigations.

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The effect of centrifugal force on the transcription levels of collagen type I and collagenase in cultured canine gingival fibroblasts

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Abstract

An orthodontically treated tooth is often destabilized in its newly corrected location and relapses towards its original position. Hitherto, the explanation for this phenomenon was that orthodontic force brings about "stretching" of gingival collagen fiber, which "pull back" the tooth towards its pretreatment position. A previous ultrastructural study showed that after force application the gingival collagen fibres were torn, laterally spaced and of increased diameter. Therefore, they could not "pull back" the tooth and be the cause of the relapse. In the present study, in order to find a more plausible explanation at the molecular level, the effect of pressure on the gene transcription of collagen type I and tissue collagenase was examined by semiquantitative, reverse transcriptase-polymerase chain reaction assay. Attached buccal gingiva was excised from anaesthetized dogs and gingival fibroblasts were grown in culture. Following application of pressure (0.167 kg/1 g cell mass), the transcription of collagen type I was increased while that of tissue collagenase was decreased. These results corroborate the ultrastructural *in vivo* findings that orthodontic force is associated with larger amounts of collagen type I in the gingiva.

Keywords: Force; Collagen; Collagenase; Gene expression

1. Introduction

Orthodontic treatment aims to change the location of abnormally positioned teeth. This is achieved by applying force (orthodontic force) to the tooth, which affects the attachment apparatus comprising the periodontal ligament, alveolar bone, cementum, and gingiva (Reitan, 1985). Tooth movement occurs as the alveolar bone remodels following the application of prolonged force. The nature of orthodontic force is both that of pressure on one side and of tension on the opposite side (Reitan, 1985). On the pressure side there is a compressive force in the direction of tooth movement causing extensive remodelling of the periodontal ligament, mainly due to collagen breakdown, and resorption of alveolar bone. On the tension side there is increased bone deposition and increased collagen synthesis in the periodontal ligament. The integrity and dimensions of the periodontal ligament are maintained

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Abbreviations: (RT)-PCR, (reverse transcriptase)-polymerase chain reaction.

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following tooth movement because of the high turnover rate of its collagen fibres.

The gingival response to orthodontic force differs from that of the periodontal ligament. The gingival tissue is compressed in the direction of the tooth movement, and the amount of collagen fibres within the compressed gingiva is increased (Boisson and Gianelly, 1981).

The success of orthodontic treatment depends on the long-term stability of the treated tooth. However, relapse in tooth position is being observed after orthodontic rotation, even after an adequate period of retention. This relapse indicates the presence of an, as yet, unexplained force. On the basis of light-microscopic studies (Reitan, 1959; Edwards, 1968), it was postulated that rotational movement brings about "stretching" of gingival collagen fibres. As these fibres connect the tooth to the gingiva, it was assumed that the "stretched" fibres pull the tooth back towards its pretreatment position. The validity of this assumption was questioned in a recent ultrastructural study on dogs (Redlich et al., 1996) in which it was shown that the gingival collagen fibres were torn, ripped, laterally spaced and increased in diameter after tooth rotation. All of these changes are incompatible with the assumption of "stretching".

Recently, an *in vivo* study showed that orthodontic pressure brought about a significant increase in the synthesis of collagen type I in the periodontal ligament (Baumann, et al., 1997). Our objective now was to test the hypothesis that pressure brings about changes in the phenotype of gingival fibroblasts *in vitro* by modulating the expression of collagen type I and collagenase. This was achieved by centrifugation of the cells in culture, thus exerting controlled pressure upon them.

2. Materials and methods

The protocols for the work with animals were approved by the Animal Care and Use Committee of the Hebrew University of Jerusalem.

Three dogs were anaesthetized with 2% Rampun (Bayer, Leverkusen, Germany) and 6% pentobarbital sodium, 0.3 ml/kg body wt (Ceva, Paris, France). Pieces of attached buccal gingiva were excised at several sites from both jaws, placed in cold Hank's solution containing heparin 10 μl/ml, streptomycin 100 μg/ml, penicillin 100 μl/ml, and cut into small fragments with a sterile scalpel. The fragments were treated with 5 ml of 0.25% trypsin for 1 hr at 37°C. After incubation the supernatant was removed and tissue fragments were treated with 5 ml of 0.1% collagenase type IV (Sigma, St. Louis, USA), 290 μl/ml, and 0.005 M calcium chloride for 2 hr. Collagenase-treated samples were sieved by gentle pressure through a sterile nylon (60) μmesh and collected in a centrifuge plate. The suspension was centrifuged, the cell pellet was washed twice in Hank's solution and once in complete medium containing Dulbecco's modified Eagle medium, 10% fetal calf serum, 2% L-glutamine, streptomycin 50 μg/ml and penicillin 50 μl/ml, and then plated into 50-ml flasks. The flasks were incubated in a humid environment of 95% air, 5% $C0_2$ to permit attachment. After attachment, 10 ml of complete medium was gently poured; the medium was changed every 2–3 days until confluence was achieved. To continue further passages the cells were removed with 0.25% trypsin and the procedure repeated, as described above, up to the third passage. A total of 30 flasks was thus obtained.

The first series of centrifugations sought to determine the conditions of pressure on the cells that would not damage their vitality. A plate onto which the flasks had been mounted was inserted into a centrifuge with a horizontal microplate rotor. Several speed/duration experiments were then conducted and the results monitored using the trypan-blue exclusion test for cell vitality. Controls consisted of cultured cells held under normal conditions but at room temperature, while the other groups were spinned.

To extract total RNA the cells were suspended in Tri-Reagent (Molecular Research Center, Cincinnati, USA), according to the manufacturer's protocol. After DNAase treatment, 10 μg of total RNA extracts were reverse-transcribed using 0.5 μg of (dT)15 as primer together with 200 units of Moloney murine leukaemia virus reverse transcriptase, according to the manufacturer's specifications (United States Biochemical, USA). Four percent of the cDNA produced was amplified in a PCR thermocycler (Perkin-Elmer/Cetus) using the following specific oligonucleotide primers:

1. Collagen type I: 5'-GCGCCAGGAGA-ACCGTCTCG-3' (sense) and 5'-CCTGGCCTTA-AGGGAATGCG-3' (antisense) for 41 cycles.
2. Collagenase: 5'-CATCCAAGCCATATATG-GACGTTCC-3' (sense) and 5'-TCTGGAG-AGTCAAAATTCTCTTCGT-3' (antisense) for 37 cycles.

PCR was performed with different numbers of cycles to ensure that amplification would occur in the linear range. Negative and positive controls were included in every run. PCR amplification was done under the following reaction conditions (final concentrations): 200 μM each dNTP, 100 pmol each primer, 1.0 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8), 0.1% Triton X-100 and 2 units of Vent polymerase (New England Biolabs, USA) in a total reaction volume of 50 μl. Temperature and duration of denaturation, annealing and extension were determined for each primer.

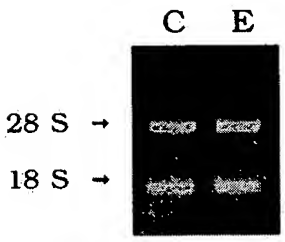

Fig. 1. A sample of total RNA extracted from control gingiva (C) and following induction of pressure (E). Similar amounts of total RNA as shown by the 18 *s* rRNA were subjected to cDNA synthesis and PCR amplification.

The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed with 667 Polaroid film. Actin mRNA was also assessed in the total RNA preparations as an internal control. Two percent of the cDNA produced was amplified by PCR for 35 cycles using specific actin oligonucleotide primers: 5'-GAGACCTTCAACACCCCAGCC-3' (sense) and 5'-GGCCATCTCTTGCTCGAAGTC-3' (antisense). The annealing temperature was set at 60°C, and the Mg concentration 1 mM. The Polaroid film was scanned and the density of the bands was computer analysed by TINA version 2 (Raytest Isotopenmebgerate, Germany) image software. The relative intensity of the bands for the different primers was divided by the intensity of the actin band.

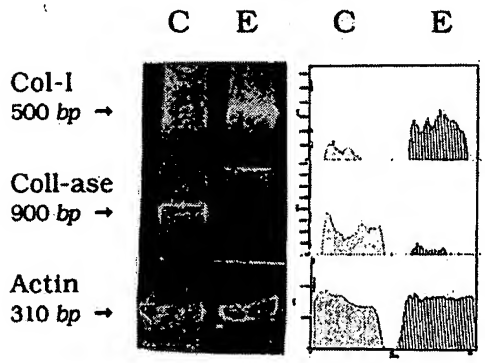

Fig. 2. Detection of collagen type I (col I) and collagenase (coll-ase) transcription in gingival fibroblasts before and after induction of pressure. PCR analysis used oligonuleotides primers specific to collagen type I and collagenase. C, before induction of pressure; E, after induction of pressure. The PCR products were separated on ethidium bromide-stained agarose gel and photographed under ultraviolet illumination. Internal control: actin mRNA. On the right-hand side, densitometric analysis of the PCR products.

3. Results

The speed/duration of centrifugation after which 90% of the cells remained vital were chosen for the experiments; these conditions were 1000 rev/min and 90 min. Increasing centrifugation speed to 1500 rev/min or doubling it to 2000 rev/min caused 30% and 50% cell death, respectively. Speed of 1000 rev/min and duration of 120 or 150 min caused 20% and 30% cell death, respectively. The pressure exerted on the cells by centrifugation at 1000 rev/min for 90 min is equivalent to a force of 0.167 kg/1 g of cell mass [according to $F = (V^2{:}R) \times M$, where F = force, V = speed, R = radius of the rotor, M = mass]. This force is within the range of common orthodontic force (Reitan, 1985).

Similar amounts of total RNA as shown by the 18 *s* rRNA were subjected to PCR amplification (Fig. 1). The RT-PCR clearly showed that the cells under pressure expressed higher levels (×4) of collagen type I mRNA and lower levels (×7) of collagenase mRNA than the control cells (Fig. 2). The effect of pressure on collagen and collagenase gene expression was independent of the number of passages and was essentially the same in all the cultures examined (10 flasks, five experiments and five controls, of each passage).

4. Discussion

Our results demonstrate that the application of pressure on gingival fibroblasts *in vitro* caused a significant increase in gene expression for collagen type I and a decrease for collagenase. These results are similar to those of an earlier *in vitro* study (Lambert et al., 1992) which showed that tension modulated the phenotype of human dermal fibroblasts, and that this regulation operated, at least in part, at a pretranslational level: thus, the steady-state concentrations of mRNAs for procollagen α1(I), procollagen α2(I) were significantly higher and the concentration of collagenase was significantly lower in fibroblasts cultured under tension.

Gingival collagen is composed mainly of type I (90%), with some type III (8%); the remaining gingival collagens are of types IV, V, VI (Narayanan and Page, 1983; Romanos et al., 1991). Therefore, here we focused first on collagen type I.

The gingiva is characterized by an inherently high rate of collagen metabolism which is important during normal function or during orthodontic tooth movement (Sodek and Ferrier, 1988). Thus, maintaining the integrity of the gingiva requires a constant equilibrium between collagen synthesis and degradation. To assess the possible role of degradation following pressure, we

also determined the transcription level of collagenase in the affected cells. Our results show that a pretranslational interference in that equilibrium took place when the cells were submitted to temporary pressure: an increase in collagen mRNA was accompanied by a decrease in collagenase mRNA which probably caused changes in the relative amount of interstitial collagen. Indeed, such modulation of the fibroblast phenotype which changes the amount of interstitial collagen has been described in the dermis and gingiva. In specimens taken from human abdominal striae alba from multiparous and obese women the diameter of collagen fibres, mostly of collagen type I, was increased relative to those in healthy skin (De Pasquale et al., 1987). It was suggested that this change in the fibroblast phenotype is related to biological functional demand due to abnormal pressure acting on the skin. A similar picture to that in skin was also seen in an ultrastructural study on both human (Franchi et al., 1989) and canine (Redlich et al., 1996) gingiva during the application of orthodontic force. There too the fibres were significantly thicker after tooth movement than in the untreated gingiva. Also, a significant increase in collagen synthesis was found in human periodontal ligament on both the tension and pressure sides during orthodontic treatment (Baumann et al., 1997). It is assumed that the force increases the relative amount of interstitial collagen, a major structural protein of the stroma, leading to an increase in tissue rigidity and thus enhancing its ability to oppose abnormal pressure.

A similar effect of pressure on increased collagen synthesis is observed in the media of blood vessels (Sudhir et al., 1993): chronically elevated arterial blood pressure, reflecting transient mechanical force, is associated with an increase in thickness of the media due to increased collagen deposition.

It is evident that mechanical force may alter biological responses. *In vivo* the mechanotransduction pathways involve extracellular components affecting the cell surface and modulating the genome via the cytoskeleton (Wang and Butler, 1993). *In vitro* the force alters the shape and the structure of the cells evoking intracellular signals that might change cell function (Watson, 1991). The *in vivo* regulatory mechanism of orthodontic force transduction is now under investigation in our laboratory.

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